Alytes, 1988, 7 (2): 53-61.

Erythrocyte size as an indicator of ploidy level in *Rana* kl. *esculenta* before and after the metamorphosis

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The mean erythrocyte length is sufficient to classify *Rana* kl. *esculenta* individuals as diploid or triploid. Because erythrocyte size increases after metamorphosis and during the first year of postmetamorphic development, criteria for ploidy determination have to be modified according to the age of the tested animals. In contrast, the size of the erythrocyte nucleus does not significantly increase during development.

INTRODUCTION

It has long been known that erythrocyte populations in Anurans are replaced during metamorphosis. Evidence for this replacement is provided by the transition from larval to adult hemoglobin in *Xenopus laevis* (JURD & MACLEAN, 1970) and *Rana catesbeiana* (MCCUTCHEON, 1936; BENBASSAT, 1974), as well as modifications in erythrocyte shape and ultrastructure in *Bufo bufo* (ANZANEL et al., 1983) and *Rana pipiens* (HOLLYFIELD, 1966), including changes of size and volume (MCCUTCHEON, 1936; HOLLYFIELD, 1966; ANZANEL et al., 1983). These studies indicate that tadpole erythrocytes usually are larger than adult erythrocytes.

Erythrocyte size often has been used as an easy mean of determining ploidy in the *Ambystoma jeffersonianum* complex (UZZELL, 1964; AUSTIN & BOGART, 1982), *Ambystoma mexicanum* (FANKHAUSER, 1945), *Xenopus* (GEORGE & LENNARTZ, 1980), *Rana* kl. *esculenta* (UZZELL & BERGER, 1975; GÜNTHER, 1977) and *Ceratophrys* species (MERCADAL, 1981).

A study of a green frog population (*Rana* kl. *esculenta* complex) in a natural pond of the Fontainebleau forest near Paris (France), revealed the presence of diploid and triploid individuals of hybrid constitution. The triploid hybrid subpopulation consists exclusively of males with an RLL constitution, i.e. one *Rana ridibunda* genome and two *Rana lessonae* genomes. The persistence of triploid males in the Fontainebleau population is assured by crosses of RLL males with RL females, owing to a particular mode of reproduction very similar to hybridogenesis (GRAF & POLLS, 1988).

GÜNTHER et al. (1979), HOTZ (1983), UZZELL et al. (1975) and BERGER & GÜNTHER

(1988) suggest that the erythrocytes of adult green frogs could be larger than those of immature specimens. While testing the erythrocyte size method for rapid determination of ploidy level in population samples, we noticed that the erythrocytes of tadpoles and young metamorphosed specimens were consistently smaller than those of adults of the same ploidy. It was therefore necessary to establish criteria allowing the determination of ploidy in individuals belonging to different age classes.

Material and methods

Diploid and triploid adults of both sexes were collected during the years 1985, 86 and 87 from a pond in Chanfroy Plain (Fontainebleau forest, near Paris). Froglets were collected just after metamorphosis from the same pond (June 30, 1987).

The genotypes of diploid and triploid individuals were determined on the basis of electrophoretic phenotypes of lactate dehydrogenase (LDH), aspartate aminotransferase (AAT), and glucosephosphate isomerase (GPI), using techniques described in Graf et al. (1977) and Graf & Müller (1979). Triploid hybrids were distinguished from diploids on the basis of gene-dosage effects visible in electrophoretic patterns of LDH (Uzzell et al., 1975; Günther & Hähnel, 1976). Some karyotypes were made to confirm the validity of this method. For the adult frogs analysis, 10 triploid *Rana* kl. *esculenta*, all of them males (there are no triploid females in the Chanfroy pond), as well 20 diploid *Rana* kl. *esculenta* (10 males, 10 females), and 5 diploid *Rana lessonae* (1 female, 4 males) were utilized.

Diploid and triploid tadpoles were obtained from selective experimental crosses (cross 4-86) or from frogs caught in amplexus (crosses 1-85 and 5-87) and allowed to lay eggs in the laboratory. Parents of each isolated clutch were identified by enzyme electrophoresis. Similarly, the genotypes of progenies from crosses 1-85 and 4-86 were determined on the basis of their electrophoretic phenotypes, whereas the progeny from cross 5-87 were assumed to have an RL constitution (one *ridibunda* genome and one *lessonae* genome) based on the genotypes of the parents. The identity of the parents and progenies of each cross are described in Table I.

Tadpoles were reared in 1.5 l. tanks at the density of 10-20 tadpoles per liter, in dechlorinated water changed once a day. The larvae were fed with a progressive diet of cooked egg yolk. Only tadpoles showing a good vitality were analysed. Classification of larval stages was made on the basis of the characteristics described by Gosner (1960).

Blood smears of adults were obtained by cutting a finger. Blood smears of tadpoles were obtained by cutting the extremity of the tail; controls were made in tadpoles by taking blood from the heart, to confirm that the size of the erythrocytes circulating in the tail did not differ from the average erythrocyte size.

Erythrocytes were measured on drawings from dried blood smears using a *camera lucida* at magnification of 1000. In adults, as well as in froglets and tadpoles, 4 measures were taken : the major and minor axes of the optical sections of the whole cell and the nucleus. Ten randomly chosen erythrocytes for each individual, and 10 individuals for each group, were considered for the statistical analysis. Only erythrocytes showing clear limits of the cytoplasm and nuclear membranes were measured. The area of an optical section through the

Cross 1-85

♀ ESCULENTA 2n (RL) x ♂ ESCULENTA 2n (RL)

↓

♀♀ RIDIBUNDA 2n (RR)

Cross 4-86

♀ ESCULENTA 2n (RL) x ♂ ESCULENTA 3n (RLL)

↓

♂♂ ESCULENTA 3n (RLL)

Cross 5-87

♀ ESCULENTA 2n (RL) x ♂ LESSONAE 2n (LL)

↓

♀♀, ♂♂ ESCULENTA 2n (RL)

Table I. – Origin of tadpoles and froglets used in this study. The patterns of inheritance in the three crosses result from the hybrid constitution of *esculenta* and the exclusion of one parental genome in the hybrids' germ cells (review in GRAF & POLLS, 1988). In cross 1-85, both RL parents clonally transmitted a R (= *ridibunda*) genome to progeny. In cross 4-86, the triploid RLL male contributed two L (= *lessonae*) genomes and the diploid RL female contributed one R genome. In cross 5-87, the diploid RL female clonally transmitted a R genome and the LL male transmitted non-clonal L genomes to progeny.

two longer dimensions of the cell was estimated as π.a.b, where a and b are one half the cell length and width.

Among the progeny from cross 4-86, two samples were separately studied with respect to the wintering period. The first was kept at 0 to 5°C in a cold room during 4 months to simulate hibernation; the animals did not eat during this period. The second group was maintained at about 20°C from December to April, and was given a normal diet (living insects).

RESULTS AND DISCUSSION

The mean values, standard deviations, and the maximal and minimal values of the four considered parameters (i.e. lengths of cell and nucleus major axis, areas of cell and nucleus optical sections) are given for each studied group in Table II. Three adult phenotypes have been distinguished (*Rana* kl. *esculenta* 2n, *Rana* kl. *esculenta* 3n, and *Rana lessonae* 2n), as well as three groups of tadpoles and froglets (*Rana* kl. *esculenta* 2n, *Rana* kl. *esculenta* 3n, *Rana ridibunda* 2n originating from the homotypic *Rana* kl. *esculenta* 2n crosses).

In all analysed diploid and triploid lineages a clear increase of the erythrocyte length

Table II. – Erythrocyte size in diploid and triploid green frogs from the Chanfroy population.

		Cell length (µm)			Nucleus length (µm)			Cell area (µm²)			Nucleus area (µm²)		
		mean	SD	range	mean	SD	range	mean	SD	range	mean	SD	range
esculenta 3n	tadp. s 44	25.0	1.6	27.5-22.2	10.6	0.5	12.5-10.0	288.8	25 0	319.9-241 3	52 6	5.0	62 3-44.8
esculenta 3n	postmetam.	25.0	0.3	25 5-24.3	11.2	0.7	12.3-10.0	297.3	18 5	323.3-274 2	55 2	8 6	68 1-43.6
esculenta 3n	before 1st hib.	27.6	1.0	29 6-26.1	11.5	0.3	12.1-11.1	365.3	21 4	400.4-333 9	48.3	3.8	55 3-43.0
esculenta 3n	hibernating	28.4	0.9	29.7-27.5	11.9	0.4	12.6-11.4	375.5	13 7	405.9-327.7	59.9	7.4	71.7-51.6
after 1st. hib.	non hib.	29.2	0.9	31.0-28.2	12.8	1.5	15 7-11.8	387.2	27 6	427.9-344.9	56.4	2.4	60.5-53.6
	hib. + n. hib.	28.8	0.9	31.0-27.5	12.4	0.9	15 7-11.4	381.3	20.7	427.9-327.7	58.1	4.9	71.7-51.6
esculenta 3n	adults	29.8	1.4	31.7-27.1	11.5	0.8	12 9- 9.9	420.7	31.5	459.2-382.9	54.9	7.6	64.9-42.2
esculenta 2n	tadp s 36	18.1	0.5	18.9-17 4	9.8	1.5	13 8- 8.6	189.5	13.3	215.5-168.3	47.5	8 6	69 7-38.5
ridibunda 2n	tadp. s. 44	20.8	1.4	22 7-18 5	9.7	0.7	10.8- 8.6	192.7	21.7	230.1-169 4	46.3	7 2	57 8-35.7
esculenta 2n	postmetam.	20.8	1.0	21.7-18.9	8.5	0.6	9.2- 7.7	209.7	16 7	230.7-184 2	38 3	5.7	47.5-28.7
	♂♂	23.3	0.7	25.4-22.9	9.0	0.6	9.8- 8.4	277.4	12 6	296.5-245.7	39.4	5.4	49.5-29.7
esculenta 2n adults	♀♀	23.9	0.8	25.0-23.0	8.9	0.6	9.6- 7.5	286 4	13.1	305.3-258.6	39.4	5.1	47.5-32 4
	♂♂ + ♀♀	23.6	0.7	25.4-22.7	9.0	0.6	9.8- 7 5	281 9	12.9	305.3-245.7	39.4	5.3	49.5-29.7
lessonae 2n	adults	24.9	2 3	28.5-22.4	9 0	1 0	10.8- 7 8	301.5	38.0	357.1-243.6	42.4	13.3	67.7-28.7

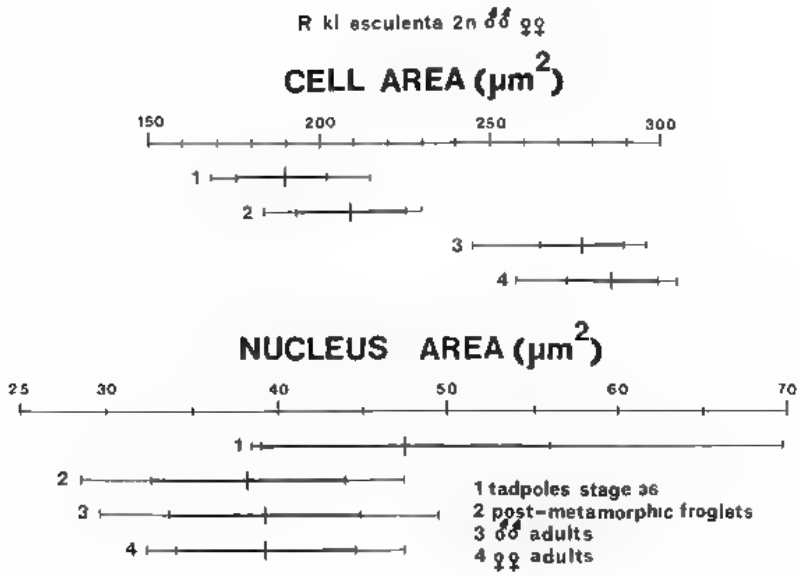

Fig. 1. – Top : means and ranges of cell areas of erythrocytes in diploid *Rana* kl. *esculenta* during ontogenesis. Bottom : means and ranges of nucleus areas of erythrocytes in diploid *Rana* kl. *esculenta* during ontogenesis.

and area was observed, from larval to adult stages during ontogenesis. This increase in erythrocyte size is illustrated in fig. 1 for diploid *Rana* kl. *esculenta* : the mean erythrocyte area varies from 189 μm^2 in tadpoles to 286 μm^2 in adult females and 277 μm^2 in adult males, with an intermediate value of 210 μm^2 just after metamorphosis. In contrast, the nucleus area decreased from 47 μm^2 in tadpoles to about 38 μm^2 in young metamorphosed frogs and in adults.

In triploid *Rana* kl. *esculenta* (RLL) the mean erythrocyte area increased from 289 μm^2 in tadpoles to 421 μm^2 in adults; the nucleus area did not vary significantly during ontogenesis in triploids (fig. 2). Interestingly, the mean erythrocyte areas found in triploids exceed by a factor of 1.5 the corresponding values of diploids. Hibernation apparently had no effect on erythrocyte replacement in triploid froglets.

Differences between tadpoles and adults were also observed with respect to erythrocyte shape: the coefficient of excentricity a/b is lower in larval erythrocytes (a/b = 1.40 for diploid tadpoles) than in adult erythrocytes (a/b = 1.56 for diploids). The mean excentricity in adult triploids is 1.68. DAVISON (1959) similarly observed that the coefficient of excentricity of *Triturus* erythrocytes was higher in triploids than in diploids.

Of practical interest is the confirmation that diploid and triploid *Rana* kl. *esculenta* are well distinguishable on the basis of the mean erythrocyte length in samples of similar age category (fig. 3, 4, 5). This discrimination is especially clear in adults (fig. 4). In addition, it is worth noting that the mean cell length and area of erythrocytes of the "good" species *Rana lessonae* (CL = 24.9 μm; CA = 301.5 μm^2) are slightly higher than the mean values found for the diploid specimens of the hybrid *Rana* kl. *esculenta* (CL = 23.6 μm; CA = 281.9 μm^2).

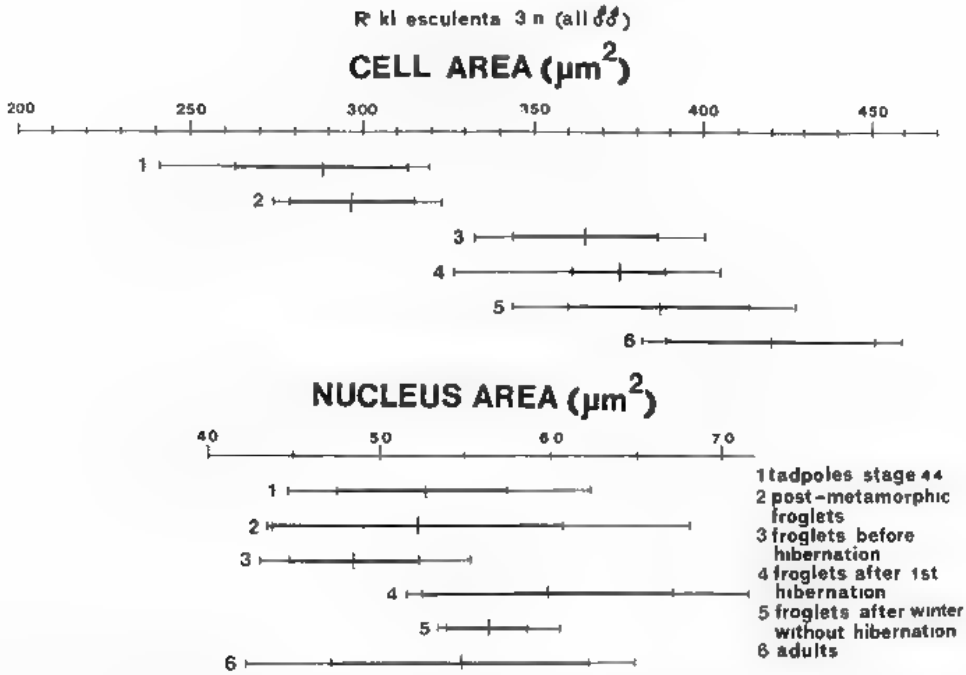

Fig. 2. – Top : means and ranges of cell areas of erythrocytes in triploid *Rana* kl. *esculenta* during ontogenesis. Bottom : means and ranges of nucleus areas of erythrocytes in triploid *Rana* kl. *esculenta* during ontogenesis.

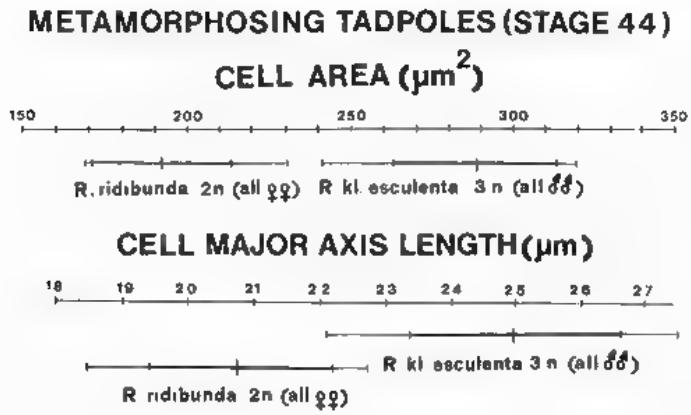

Fig. 3. – Diagram of erythrocyte size in diploid and triploid tadpoles.

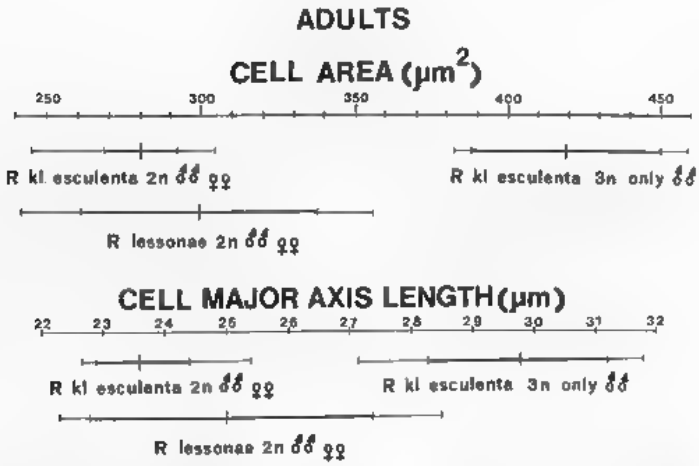

Fig. 4. – Diagram of erythrocyte size in adults of *Rana* kl. *esculenta* (diploid and triploid) and *Rana lessonae*.

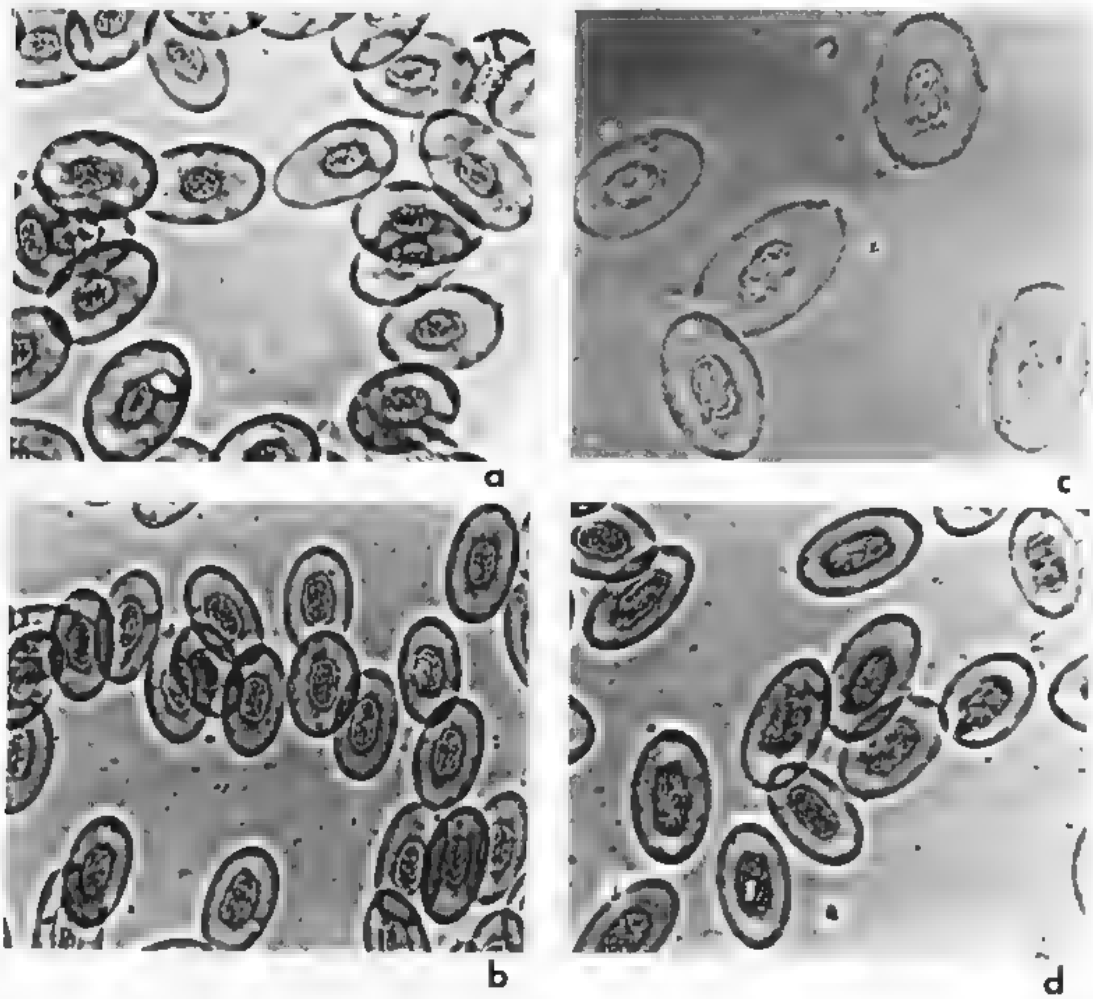

Fig. 5. – Photomicrographs (×675) of erythrocytes of *Rana* kl. *esculenta* : a. – diploid adults, b. – diploid postmetamorphic froglets, c. – triploid adults, d. – triploid postmetamorphic froglets.

Acknowledgements

Wild animals were collected with a permission number 87169 of the Direction de la Nature in France. We thank Dr. A. Dubois (Paris) and other members of the French Batrachological Society (S.B.F.) for helping in the field work. Dr. H. Tunner identified by electrophoresis and karyotypes the parents of cross 1-85. We thank Mrs. Yvette Develey for typing the manuscrit, and Mr. Alex Portianucha for preparing the figures.

Résumé

La longueur moyenne des érythrocytes constitue un paramètre suffisant pour distinguer, dans le complexe de *Rana* kl. *esculenta*, les spécimens triploïdes des spécimens diploïdes. Cependant, étant donné que la taille des érythrocytes augmente pendant la métamorphose et la première année de développement post-métamorphique, le critère de discrimination doit être modifié en fonction de l'âge des individus étudiés. La surface du noyau des érythrocytes n'augmente pas significativement au cours du développement.

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